Cell Metabolism
Article

Drosophila NPC1b Promotes an Early Step in Sterol Absorption from the Midgut Epithelium

Stephen P. Voght,[1] Megan L. Fluegel,[1,2] Laurie A. Andrews,[1] and Leo J. Pallanck[1,*]
[1] Department of Genome Sciences
[2] Molecular and Cellular Biology Graduate Program, University of Washington, Seattle, WA 98195, USA
*Correspondence: pallanck@u.washington.edu
DOI 10.1016/j.cmet.2007.01.011

SUMMARY

The NPC1 family of proteins plays crucial roles in the intestinal absorption and intracellular trafficking of sterols. The *Drosophila* genome encodes two NPC1 homologs, one of which, NPC1a, is required for intracellular sterol trafficking in many tissues. Here we show that the other *Drosophila* NPC1 family member, NPC1b, is expressed in the midgut epithelium and that NPC1b is essential for growth during the early larval stages of development. *NPC1b* mutants are severely defective in sterol absorption, and the midgut epithelium of *NPC1b* mutants is deficient in sterols and sterol trafficking intermediates. By contrast, *NPC1a* mutants absorb sterols more efficiently than wild-type animals, and, unexpectedly, *NPC1b;NPC1a* double mutants absorb sterols as efficiently as wild-type animals. Together, these findings suggest that *NPC1b* plays an early role in sterol absorption, although sterol absorption continues at high efficiency through an *NPC1a*- and *NPC1b*-independent mechanism under conditions of impaired intracellular sterol trafficking.

INTRODUCTION

Vertebrates acquire cholesterol from their diet and through the synthesis of cholesterol from acetate. Although much is known of the enzymology of cholesterol biosynthesis (Brown and Goldstein, 1986), far less is known of the molecular mechanisms by which dietary cholesterol is absorbed by the intestine. However, recent genetic and pharmacological work indicates that the Niemann-Pick C1-like 1 (NPC1L1) protein plays an important role in the intestinal absorption of dietary sterols. In particular, cholesterol absorption is largely inhibited by the drug ezetimibe, which binds to and presumably inactivates the NPC1L1 protein (Garcia-Calvo et al., 2005). Moreover, *NPC1L1* knockout mice absorb dietary cholesterol inefficiently and show no response to ezetimibe (Altmann et al., 2004; Davis et al., 2004). While these findings clearly demonstrate that NPC1L1 promotes dietary cholesterol absorption, the specific mechanism by which it does so remains unclear.

The *NPC1L1* gene is homologous to the Niemann-Pick type C1 (*NPC1*) gene, loss-of-function mutations of which cause Niemann-Pick type C disease, an autosomal recessive lipid-storage disorder. The *NPC1* and *NPC1L1* gene products are both 13-pass transmembrane proteins with a putative sterol-sensing domain, a cysteine-rich domain, broad homology to the Patched morphogen receptor, and structural similarity to the RND family of prokaryotic permeases (Carstea et al., 1997; Davies et al., 2000a, 2000b). NPC1 also contains a lysosomal targeting motif and localizes to an endosomal compartment where it promotes intracellular trafficking of cholesterol and sphingolipids, perhaps by directing the movement of sterol-rich transport vesicles to their proper locations (Ko et al., 2001) or by facilitating the export of sterols from the lysosome (Ioannou, 2000).

Although the structural similarity of NPC1L1 to NPC1 suggests that these proteins play conserved biochemical roles, many important questions concerning the function of NPC1L1 remain unanswered. For example, it remains unclear whether NPC1L1 specifically promotes the absorption of sterols or whether this protein also participates in the absorption of other dietary factors. The identities of key factors that regulate NPC1L1 and that function in concert with NPC1L1 to promote cholesterol absorption in the intestine also remain unknown. Whether NPC1L1 promotes an early step in sterol acquisition and/or, like NPC1, a later step in intracellular trafficking of cholesterol in the intestinal epithelium also remains controversial (Sane et al., 2006; Yu et al., 2006). Finally, the precise molecular functions of both NPC1L1 and NPC1 remain unknown and the topic of much debate (Davies and Ioannou, 2006; Garver and Heidenreich, 2002; Karten et al., 2006; Malathi et al., 2004; Mellon et al., 2004).

To study the functions of the *NPC1* gene family and the mechanisms of sterol absorption and intracellular sterol trafficking, we used a genetic approach in the fruit fly *Drosophila melanogaster*. Although insects lack the ability to synthesize sterols, dietary sterols are required for proper insect development, and previous work suggests that sterol trafficking mechanisms are highly conserved in insects and vertebrates (Rodenburg and Van der Horst, 2005). The *Drosophila* genome contains two *NPC1* homologs, designated *NPC1a* and *NPC1b*. The *NPC1a* and *NPC1b* genes encode polypeptides with 42% and 35%

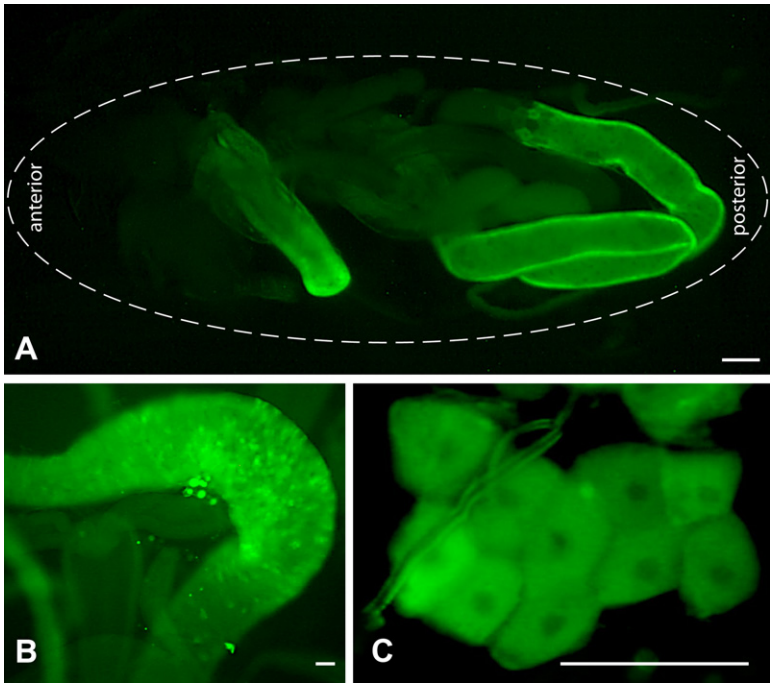

Figure 1. NPC1b Is Expressed in the Midgut Epithelium

Approximately 800 bp of sequence upstream of the *NPC1b* start codon was joined to the GAL4 transcription factor. Larvae bearing this transgene and a GAL4-responsive GFP transgene express GFP in tissues in which the *NPC1b* promoter is active.

(A) Dissected third-instar larvae, showing two distinct areas of GFP expression within the midgut. Dotted line shows approximate location of cuticle prior to dissection.

(B) Isolated midgut from 3-day-old adult fly. One area of GFP expression is detected in adult flies.

(C) Close-up of isolated GFP-expressing larval midgut cells, showing hexagonal morphology characteristic of epithelial cells. Scale bars in all images = 50 μm.

amino acid identity, respectively, to the human NPC1 protein and 34% and 29% identity, respectively, to the human NPC1L1 protein. We and others have previously demonstrated that NPC1a is ubiquitously expressed and is required for efficient intracellular sterol trafficking in many tissues, including the ring gland, where the major insect steroid hormone ecdysone is synthesized (Fluegel et al., 2006; Huang et al., 2005).

In our current work, we subjected the *NPC1b* gene to mutational analysis and found that NPC1b functions in a role homologous to that of mammalian NPC1L1 in the regulation of dietary sterol uptake from the gut epithelium. Our results suggest that NPC1b promotes an early step in sterol absorption and may participate in the absorption of other essential dietary factors. Our findings also suggest the existence of an NPC1a- and NPC1b-independent mechanism of sterol absorption.

RESULTS

Expression Analysis of NPC1b

To determine where NPC1b is expressed, we generated a reporter construct consisting of an *NPC1b* promoter-containing fragment placed upstream of the yeast GAL4 protein coding sequence. Transgenic flies bearing this *NPC1b-GAL4* fusion construct were crossed to flies carrying a *UAS-GFP* transgene. Larval progeny of this cross that bear both the *NPC1b-GAL4* and *UAS-GFP* transgenes express GFP in two distinct areas, corresponding to compartments m2 and m10–m12 of the midgut (Figure 1A; see also Figure S1 in the Supplemental Data available with this article online; Murakami et al., 1999). GFP expression diminishes during pupation and then reappears in the adult midgut approximately 2 days posteclosion (Figure 1B). Higher magnification of the larval midgut revealed that the *NPC1b-GAL4* transgene drives expression specifically in the midgut epithelium and not in surrounding muscle tissue (Figure 1C). GFP fluorescence was not detected in any tissues outside of the midgut at any stage of development, suggesting that NPC1b expression is restricted to the midgut epithelium throughout the *Drosophila* life cycle. Control animals bearing only the *NPC1b-GAL4* or *UAS-GFP* transgene failed to produce detectable GFP fluorescence (data not shown), demonstrating that the *NPC1b* promoter is directly responsible for GFP expression in the midgut.

Identification of *NPC1b* Mutants

To investigate the functional role of NPC1b, we subjected the *NPC1b* gene to mutational analysis. An *NPC1b* mutant allele was created using a gene-targeting construct that included a nonsense mutation in the first exon of *NPC1b* at codon 392, several nucleotide alterations in the first intron designed to eliminate splicing, a diagnostic restriction site, and other alterations required for gene targeting (Rong and Golic, 2000). This construct was used to generate mutations as detailed in the Experimental Procedures section and outlined schematically in Figure 2A. From a screen of 35,000 flies, we recovered two independent lines in which a targeting event occurred. Both lines carried a wild-type copy of the *NPC1b* gene and a tandem copy bearing the inactivating mutations (Figure 2A). Subsequent generation of a double-stranded break between the mutationally altered and wild-type copies of the *NPC1b* gene led to the recovery of 33 recombinant lines bearing only the mutant copy of *NPC1b*. Loss of wild-type *NPC1b* and retention of the mutation were verified by PCR across the diagnostic restriction site introduced

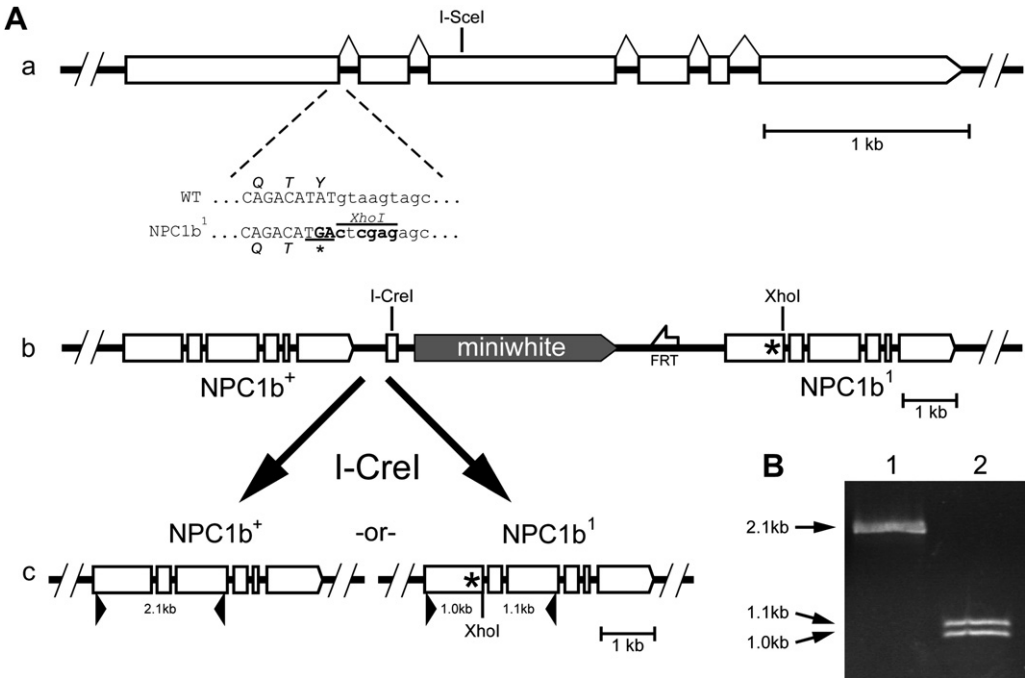

Figure 2. Generation of an *NPC1b* Null Mutation

(A) An *NPC1b* genomic fragment was modified to include a stop codon at amino acid position 392, several splice-site mutations, an XhoI diagnostic site, and an I-SceI restriction site (Aa). After insertion into the *Drosophila* genome, the construct was subsequently excised as a linear fragment via the flanking FRT sites and the I-SceI site. This linear fragment can recombine at the *NPC1b* genomic locus, resulting in tandem integration, with the wild-type copy of the gene followed by the *miniwhite* transgene, an I-CreI restriction site, and the targeted allele (Ab). The tandem duplication was resolved to a single copy by generating a double-stranded break at the I-CreI site, followed by recombination-mediated repair, leaving either the wild-type *NPC1b* gene or the targeted allele of *NPC1b* with the inactivating mutations (Ac).

(B) PCR amplification using primers flanking the inactivating mutation (arrowheads in Ac), followed by digestion with the XhoI restriction enzyme, results in a 2.1 kb fragment if the wild-type gene sequence is present (lane 1) or 1.1 and 1.0 kb digested fragments if the targeted allele is present (lane 2).

into the targeting construct (Figure 2B). Initial characterization indicated that all of these lines were molecularly and phenotypically identical, so one line, designated *NPC1b*^{1}, was chosen for detailed characterization.

NPC1b is located on the X chromosome, and both hemi- and homozygous *NPC1b*^{1} mutants died at the second-instar larval stage of development. Mutant larvae showed no gross morphological or behavioral defects and were able to survive for at least a week as second-instar larvae, while heterozygous and nonmutant siblings progressed through development normally (Figure 3). Approximately 5% of mutant larvae arrested at the molt from second to third instar, as evidenced by the appearance of a few dead larvae with duplicated mouth hooks and spiracles. To confirm that the recessive lethal phenotype of *NPC1b*^{1} mutants derives specifically from loss of NPC1b function, several experiments were performed. First, we tested whether the deficiency chromosomes Df(1)C1 and Df(1)A118, which bear deletions of the *NPC1b* region, were able to complement the *NPC1b*^{1} recessive lethal phenotype. The *NPC1b*^{1} mutation in *trans* to these deficiency chromosomes conferred a phenotype identical to *NPC1b*^{1} hemi- or homozygous mutants. Second, we generated a genomic construct consisting of the entire coding sequence of the *NPC1b* gene and 860 base pairs of upstream sequence. When introduced into an *NPC1b*^{1} mutant background, this construct fully rescued the *NPC1b* recessive lethal phenotype to adulthood in expected Mendelian ratios. The rescued flies were fertile and showed no distinguishable phenotypes or shortened life span, indicating that this construct bears all of the transcriptional elements necessary for proper NPC1b function. Third, we used the *NPC1b-GAL4* construct to induce expression of a GAL4-responsive *NPC1b* construct (designated *UAS-NPC1b*) consisting of the *NPC1b* locus lacking all upstream promoter sequences. This combination of transgenes was sufficient to completely rescue the *NPC1b*^{1} mutant phenotype. Together, these results demonstrate that the phenotypes associated with *NPC1b*^{1} result specifically from loss of NPC1b function. Moreover, our finding that the lethal phase of *NPC1b*^{1} homozygotes is indistinguishable from that of animals bearing the *NPC1b*^{1} allele in *trans* to a deletion of the *NPC1b* region indicates that the *NPC1b*^{1} mutation represents a null allele of the *NPC1b* gene.

In an effort to identify additional *NPC1b* alleles, we tested whether other mutations mapping to the *NPC1b* region could complement the *NPC1b*^{1} mutation. One mutation, *NPC1b*$^{R9\text{-}28}$ (Perrimon et al., 1989), failed to complement the *NPC1b*^{1} recessive lethal phenotype. *NPC1b*^{1}/*NPC1b*$^{R9\text{-}28}$ transheterozygotes died as second-instar

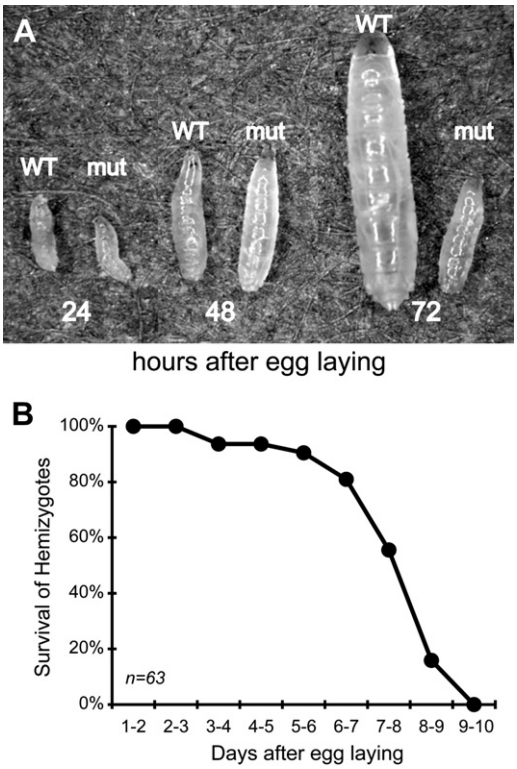

Figure 3. *NPC1b* Mutants Fail to Progress through Development

(A) Comparison of size differences between *NPC1b*^{1} hemizygous (mut) and isogenic wild-type control (WT) larvae. Larvae were genotyped at hatching and photographed at the indicated time points.

(B) Hemizygous mutant larvae (n = 63) were collected at hatching and assayed every 24 hr for survival. Though most larvae survive for an extended period of time, they do not develop beyond the second-instar stage.

larvae and, like *NPC1b*^{1} mutant larvae, were able to survive for at least a week at this developmental stage. Sequencing of the *NPC1b*$^{R9\text{-}28}$ allele revealed a stop-codon mutation at amino acid position 289 in the *NPC1b* coding sequence, confirming that this mutation is an allele of the *NPC1b* gene. The *NPC1b*$^{R9\text{-}28}$ mutation conferred lethality at the second-instar larval stage of development, and this lethality was rescued by both the *NPC1b* genomic rescue construct and the *NPC1b-GAL4* transgene in conjunction with the *UAS-NPC1b* transgene. These findings indicate that the *NPC1b*$^{R9\text{-}28}$ allele is, like *NPC1b*^{1}, a null allele of the *NPC1b* gene.

Sterol Absorption Analysis in *NPC1b* Mutants

Insects are unable to synthesize sterols from acetate and thus require a dietary source of sterols for the synthesis of the steroid molting hormone ecdysone, which is required for progression of larval development and metamorphosis. Given the restriction of expression of NPC1b to the midgut and the failure of *NPC1b* mutants to molt to the third-instar larval stage, we hypothesized that *NPC1b* mutants may be deficient in ecdysone production due to a primary defect in the absorption of a dietary sterol. Several experiments were performed to explore this hypothesis. Because the lethal phase of *NPC1a* mutants is significantly extended by either increased dietary sterol content or dietary ecdysone (Fluegel et al., 2006; Huang et al., 2005), we first tested whether similar treatment of *NPC1b* mutants would also extend their lethal phase. Dietary supplementation had no influence on the *NPC1b* lethal phase, even when using feeding paradigms sufficient to rescue *NPC1a* mutants to the adult stage of development (data not shown). Moreover, feeding *NPC1b*^{1} heterozygous females a high-cholesterol diet in an effort to induce increased sterol deposition into developing eggs failed to influence the lethal phase of their *NPC1b*^{1} hemizygous offspring (data not shown).

Our inability to detect an effect of dietary cholesterol or ecdysone on the lethal phase of *NPC1b* mutants may reflect an absolute block in sterol absorption in these mutants. To test this hypothesis, we compared the sterol abundance of *NPC1b* mutants and wild-type controls, but we were unable to detect any significant difference in total sterol levels between these genotypes (Figure S2). However, previous work indicates that substantial amounts of sterol are deposited maternally in insects (Gilby, 1965), which may mask our ability to detect a defect in dietary sterol absorption in first-instar larvae. To address this possibility, we developed an assay that selectively measures the efficiency of dietary cholesterol absorption in larvae. This analysis was performed by collecting larvae shortly after hatching and placing them on food containing [^{3}H]cholesterol and [^{14}C]glucose. Radioactive glucose, which is acquired through a mechanism distinct from cholesterol, was included to allow normalization of cholesterol absorption relative to glucose to control for possible differences in the rate of eating between genotypes. Larvae were given 18 hr to consume labeled food and then transferred to unlabeled food for another 4 hr to purge unabsorbed gut contents. Experiments using dyes that cannot be absorbed from the larval midgut demonstrated that wild-type larvae as well as *NPC1a* and *NPC1b* null mutants completely dispel unabsorbed gut contents within this time period (data not shown). At the end of this time period, the [^{3}H]cholesterol and [^{14}C]glucose content of larval homogenates was determined. Results of this analysis indicated that the cholesterol:glucose ratio was severely and equally reduced in *NPC1b*^{1} and *NPC1b*$^{R9\text{-}28}$ mutants relative to wild-type (Figure 4). By contrast, *NPC1a* null mutants, which we previously showed have a slightly elevated total sterol content (Fluegel et al., 2006), exhibited a significantly increased cholesterol:glucose ratio relative to wild-type (Figure 4). Because there was no statistically significant difference in glucose uptake between genotypes, and because pulse-chase experiments indicated that there was little or no turnover of cholesterol over the time course of our analysis (data not shown), our findings cannot be explained by alterations in glucose absorption or altered metabolism of glucose or cholesterol. Rather, our results are best explained by decreased cholesterol absorption

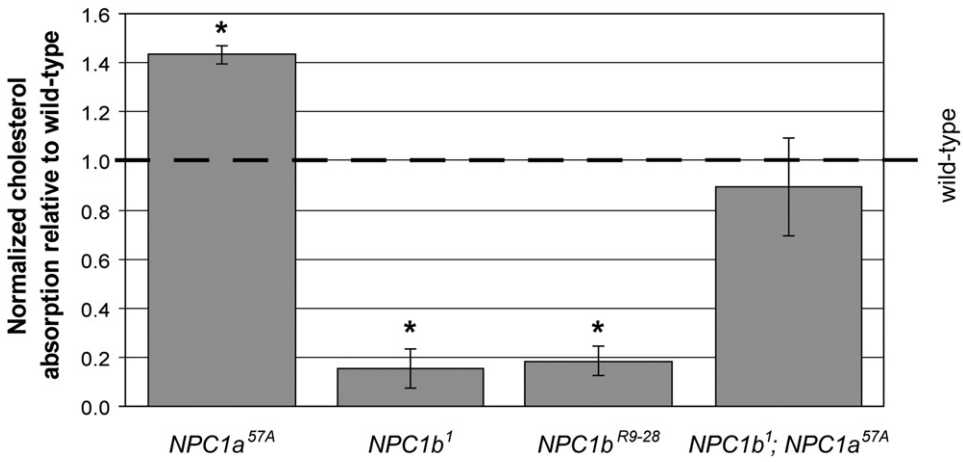

Figure 4. Cholesterol Absorption Is Severely Impaired in *NPC1b* Mutants and Increased in *NPC1a* Mutants

[^{3}H]cholesterol and [^{14}C]glucose absorption was assessed in first-instar larvae, and the cholesterol absorption values were normalized to glucose absorption. The histogram displays the cholesterol:glucose ratios for each genotype analyzed relative to the cholesterol:glucose ratio of wild-type larvae. Each value represents the mean ± standard deviation of at least three independent experiments to measure cholesterol and glucose absorption. *$p < 0.005$ by Student's t test.

efficiency in *NPC1b* mutants and increased cholesterol absorption efficiency in *NPC1a* mutants.

If NPC1b is essential for dietary sterol absorption, we would predict that the $NPC1b^1$ sterol absorption defect would be epistatic to the increased sterol absorption phenotype of $NPC1a^{57A}$ mutants. To test this prediction, we created $NPC1b^1;NPC1a^{57A}$ double mutants to measure their sterol absorption. $NPC1b^1;NPC1a^{57A}$ double mutants were recovered in Mendelian numbers and died primarily during the first-instar larval stage of development. This lethal phase was indistinguishable from that of $NPC1a^{57A}$ mutants and indicates that loss of *NPC1a* is epistatic to loss of *NPC1b* in terms of lethal phase. In contrast to our prediction, $NPC1b^1;NPC1a^{57A}$ double mutants appeared to efficiently absorb sterols and were not significantly different from wild-type larvae in this capacity (Figure 4). While $NPC1b^1;NPC1a^{57A}$ double mutants did not absorb sterols as efficiently as $NPC1a^{57A}$ single mutants, our findings indicate that the sterol absorption phenotype of $NPC1a^{57A}$ mutants is epistatic to the $NPC1b^1$ phenotype. Our findings also demonstrate that larvae retain the ability to efficiently absorb sterols in the complete absence of NPC1a and NPC1b.

Studies of Intracellular Sterol Trafficking in *NPC1b* Mutants

Our findings that the essential functions of NPC1b reside in the midgut and that *NPC1b* mutants have defective sterol absorption closely parallel findings in studies of NPC1L1, a vertebrate homolog of NPC1b. These similarities raise the possibility that NPC1b and NPC1L1 act through identical mechanisms to promote dietary sterol absorption. However, the mechanism by which NPC1L1 promotes sterol absorption remains controversial. One possibility is that NPC1L1 promotes an early event, perhaps at the plasma membrane, to allow the entry of dietary sterols into intestinal epithelial cells (Altmann et al., 2004); alternatively, NPC1L1 may play a later role in promoting intracellular trafficking of dietary sterols, similar to NPC1 (Davies et al., 2005). In an effort to distinguish these models, we compared the distribution of sterols in the midgut epithelium of *NPC1a* and *NPC1b* mutants using the fluorescent cholesterol-binding compound filipin. Midgut tissues from *NPC1a* mutants, *NPC1b* mutants, and wild-type controls were dissected and stained in parallel. As expected from previous work (Huang et al., 2005), results of this study revealed an accumulation of sterol-rich trafficking organelles in the midgut epithelium of *NPC1a* mutants (Figure 5). By contrast, midgut tissues from *NPC1b* mutants appeared to be largely devoid of filipin staining relative to wild-type and did not display an accumulation of sterol-rich trafficking organelles (Figure 5). To test whether mutations in *NPC1b* block the accumulation of sterol-rich trafficking organelles that are observed in the midgut of *NPC1a* mutants, we analyzed the distribution of sterols in *NPC1b;NPC1a* double mutants. Results of this analysis indicated that midgut tissues from *NPC1b;NPC1a* double mutants were, like *NPC1b* mutants, devoid of sterol and failed to accumulate sterol trafficking intermediates. Together, these findings suggest that the diminished sterol absorption in *NPC1b* mutants derives from an early defect in sterol absorption rather than a block in intracellular trafficking of cholesterol and that loss of NPC1b function in the midgut is epistatic to the NPC1a sterol trafficking defect in this tissue.

Previous work suggests that intracellular sterol trafficking continues at reduced efficiency in *NPC1a* null mutants in at least some tissues (Fluegel et al., 2006; Huang et al., 2005). One possible explanation for this finding is that other factors act redundantly with NPC1a to promote intracellular sterol trafficking. Although use of the *NPC1b-GAL4* reporter construct indicates that NPC1b

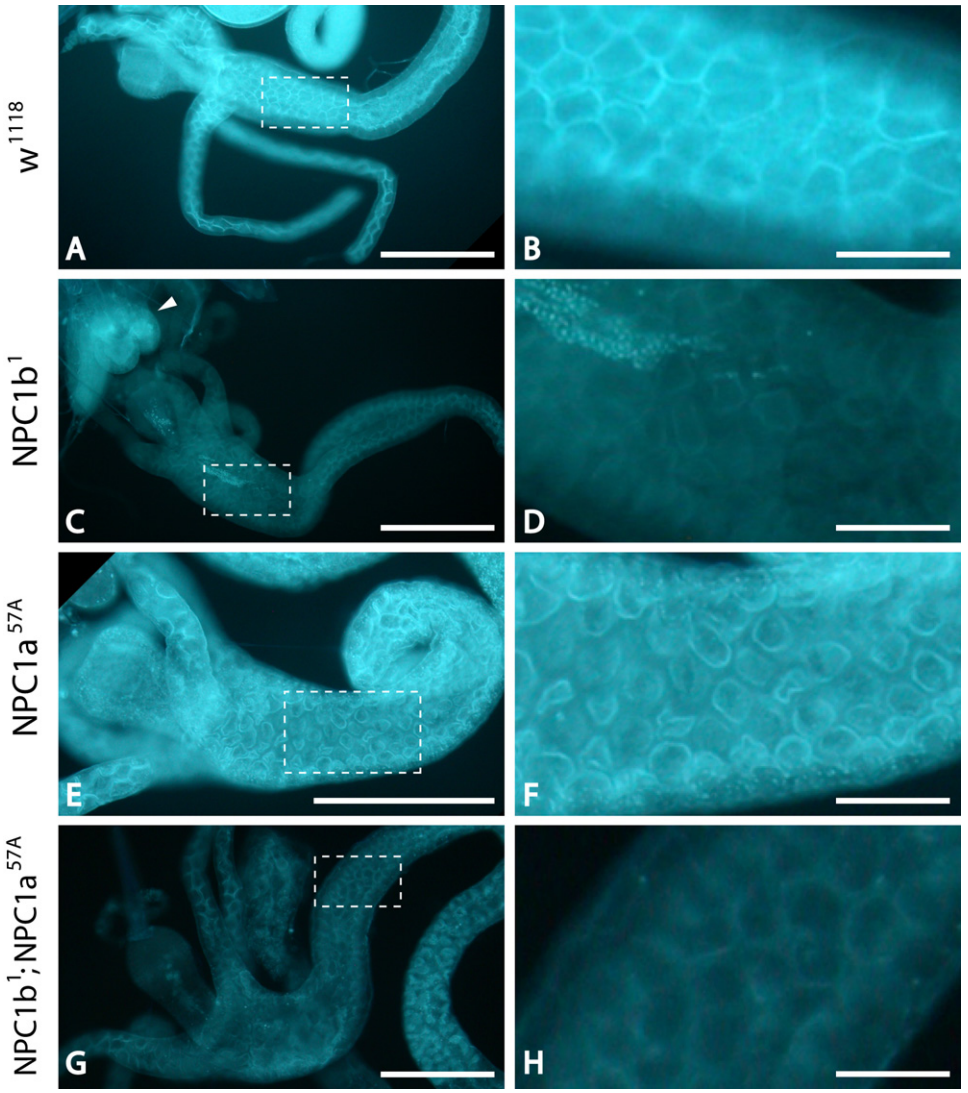

Figure 5. *NPC1b* Mutants Show Dramatic Absence of Midgut Sterols

Age-matched wild-type (A and B), *NPC1b*^{1} mutant (C and D), *NPC1a*^{57A} mutant (E and F), and *NPC1b*^{1};*NPC1a*^{57A} double mutant (G and H) 12–24 hr old larval midguts were stained with filipin in order to assess total sterol levels. Images show protoventriculus, gastric cecae, and the first two segments of the midgut. Boxes in (A), (C), (E), and (G) denote areas of magnification in (B), (D), (F), and (H), respectively. *NPC1b*^{1} and *NPC1b*^{1};*NPC1a*^{57A} midgut tissues show dramatically less filipin fluorescence than *NPC1a*^{57A} and wild-type controls, with no accumulation of sterol trafficking intermediates. *NPC1a*^{57A} mutant midgut tissues show sterol trafficking intermediates. Arrowhead in (C) denotes *NPC1b*^{1} larval CNS, which shows wild-type intensities of filipin staining. Clusters of small punctate spots in (C) and (D) are undigested yeast within the midgut lumen. Images were taken at the same exposure settings. Scale bars = 50 μm in (A), (C), (E), and (G) and 10 μm in (B), (D), (F), and (G).

expression is restricted to the midgut, we cannot exclude the possibility that our system fails to fully recapitulate the NPC1b expression pattern or that NPC1b expression is altered in an *NPC1a* null mutant. Therefore, to test the hypothesis that NPC1b normally plays a role in intracellular sterol trafficking in peripheral tissues or is activated to perform this role in an *NPC1a* mutant, we compared the filipin staining pattern in extra-gut tissues from *NPC1a* mutants, *NPC1b* mutants, *NPC1b;NPC1a* double mutants, and wild-type controls. These studies failed to detect differences in filipin staining in peripheral tissues from *NPC1b* mutants and wild-type controls (Figure 6). Furthermore, the redistribution of sterol into trafficking intermediates that was observed in peripheral tissues of *NPC1a* mutants appeared to be indistinguishable from that observed in *NPC1b;NPC1a* double mutants. We also tested whether transgenic expression of NPC1b could substitute for NPC1a and restore sterol trafficking in *NPC1a* mutants. However, we were unable to rescue the *NPC1a* recessive lethal phenotype by driving expression of NPC1b in tissues known to require NPC1a. Together, these findings indicate that NPC1b plays no role in intracellular sterol trafficking in peripheral tissues and that this factor is incapable of substituting for NPC1a.

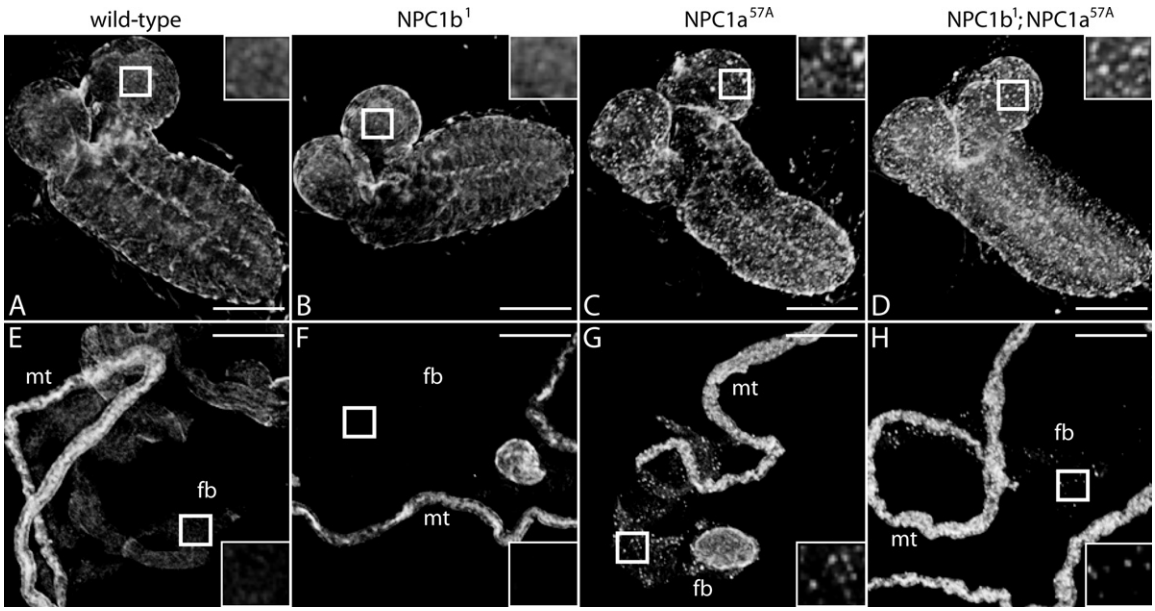

Figure 6. Sterol Trafficking Intermediates Accumulate in *NPC1a* Mutants and *NPC1b;NPC1a* Double Mutants, but Not in *NPC1b* Mutants

First-instar larval tissues were stained with filipin in order to assess cholesterol trafficking. In both wild-type controls and $NPC1b^{1}$ tissues, the sterol distribution appears to be uniform. In $NPC1a^{57A}$ and $NPC1b^{1};NPC1a^{57A}$ larvae, sterols accumulate in a punctate pattern throughout the tissues analyzed. Scale bars = 50 μm in (A)–(D) and 25 μm in (E)–(H).

(A–D) Larval brains.

(E–H) Malpighian tubules (mt) and fat body (fb) isolated from larvae of the indicated genotype.

DISCUSSION

The genomes of humans, mice, flies, and worms each encode a pair of closely related NPC1 homologs. While this finding raises the possibility that the NPC1 paralogs in metazoans have partitioned into specific evolutionarily conserved functional roles, molecular and functional studies have not completely supported this conclusion. For example, worms, flies, mice, and humans each have one NPC1 family member that appears to promote intracellular sterol and lipid trafficking in a broad set of tissues and another NPC1 family member that is expressed in a more restricted fashion (Altmann et al., 2004; Davis et al., 2004; Sym et al., 2000). However, the expression pattern of the more restricted NPC1 family member is not conserved. In particular, murine NPC1L1 is expressed predominantly or exclusively in the intestine, where it functions in sterol absorption. By contrast, human NPC1L1 is expressed most highly in liver, suggesting a hepatic function for this protein in addition to its well-documented role in dietary cholesterol absorption (Davies et al., 2005). An even greater difference is seen in the expression of *C. elegans* NCR-2, which is restricted to a pair of neuroendocrine cells and the somatic gonad (Li et al., 2004; Sym et al., 2000). Our current study indicates that *Drosophila* NPC1b is exclusively required in the midgut, where it plays a major role in the absorption of dietary sterols. These features most closely resemble those of murine NPC1L1 and, together with previous work, strongly suggest that *Drosophila* NPC1a and NPC1b provide intracellular sterol trafficking and sterol absorption functions that are equivalent to vertebrate NPC1 and NPC1L1, respectively.

Our finding that *Drosophila* NPC1b provides a function that is apparently equivalent to vertebrate NPC1L1 afforded us the opportunity to address conflicting models regarding the role of NPC1L1 in cholesterol absorption. Previous work has generated disagreements on the subcellular distribution of NPC1L1: Some studies suggest that it localizes to the plasma membrane, others suggest that it localizes to internal endosomal compartments, and still others suggest that it translocates between compartments as part of a sterol transporter activity (Davies and Ioannou, 2006; Iyer et al., 2005; Sane et al., 2006; Yu et al., 2006). These findings raise questions as to whether NPC1L1 promotes an early step in sterol absorption at the plasma membrane and/or a later step in the intracellular trafficking of sterol-rich endocytic trafficking intermediates. Our analysis of midgut tissues from *NPC1b* mutants supports a model in which NPC1b promotes an early role in the absorption of sterols at the plasma membrane. In particular, our findings that the midgut epithelium from *NPC1b* mutants appears to be devoid of intracellular accumulations of sterol in transport organelles and that the midgut epithelium is largely depleted of sterol suggest that NPC1b acts at an early step in sterol absorption. However, we cannot exclude the possibility that NPC1b plays an additional role in promoting the intracellular trafficking of sterol-enriched transport organelles following its

early role in sterol absorption. Given the similar biological functions of NPC1b and NPC1L1, we propose that NPC1L1 also acts at an early step in sterol absorption within the small intestine.

While our current data suggest that the *Drosophila NPC1a* and *NPC1b* genes provide functions equivalent to the vertebrate *NPC1* and *NPC1L1* genes, respectively, the consequences of mutations in these genes are not equivalent in flies and vertebrates. In particular, mutations in the *Drosophila NPC1* family members result in recessive lethal phenotypes at an early stage of development. In the case of *NPC1a*, this lethality is due to a failure in cholesterol trafficking, leading to decreased production of ecdysone, the steroid hormone responsible for molting and metamorphosis in insects (Garen et al., 1977). Null mutations in the vertebrate *NPC1* gene also lead to a failure in cholesterol trafficking and severe phenotypes in youth, but they do not lead to defects in endocrine steroidogenesis, as evidenced by the fact that mutations in the murine *NPC1* gene do not affect the concentrations of several major circulating steroid hormones (Xie et al., 2006). These differences likely reflect the fact that insects are sterol auxotrophs (Clayton, 1964), whereas vertebrates are able to synthesize sterols from acetate and therefore rely less on dietary cholesterol, and perhaps the efficiency of intracellular sterol trafficking. The absolute requirement for sterols in the *Drosophila* diet may also offer an explanation for the recessive lethal phenotype of *Drosophila NPC1b* mutants relative to the lack of effect of *NPC1L1* mutations on adult viability in vertebrates. However, as discussed more fully below, the effects of *NPC1b* mutations on viability are not fully understood at present and will require further work.

Our work on *Drosophila* NPC1a and NPC1b indicates that these proteins play nonredundant and noninterchangeable roles. In particular, the intracellular sterol trafficking defects seen in *NPC1a* mutants are not observed in *NPC1b* mutants, and the sterol absorption defect of *NPC1b* mutants is not a feature of the *NPC1a* mutants. Moreover, *NPC1b;NPC1a* double mutants do not appear to have more severe intracellular sterol trafficking and sterol absorption defects than the *NPC1a* and *NPC1b* single mutants, respectively. Our transgenic experiments further demonstrate that *NPC1a* cannot substitute for *NPC1b* (data not shown) and that *NPC1b* cannot substitute for *NPC1a*. The noninterchangeable feature of the NPC1a and NPC1b proteins may derive from differences in the subcellular localization of these factors, the requirement for other tissue- and NPC1-specific cofactors, or the possibility that these factors influence sterol trafficking in unique ways. Further experiments will be required to distinguish these possibilities.

Although our sterol absorption assay was primarily developed to explore the function of NPC1b, valuable insights into the regulation and mechanism of sterol absorption were also revealed in our sterol absorption studies of *NPC1a* mutants. For example, our results indicate that loss of NPC1a activity results in significantly increased sterol absorption. This finding is consistent with our previous work demonstrating that the sterol content of *NPC1a* mutants exceeds that of wild-type controls (Fluegel et al., 2006) and indicates that the increased sterol content of *NPC1a* mutants derives from increased sterol absorption as opposed to an alteration in sterol turnover. Surprisingly, the upregulation of sterol absorption that occurs in *NPC1a* mutants appears to be epistatic to the sterol absorption defect of *NPC1b* mutants, as evidenced by the finding that *NPC1b;NPC1a* double mutants absorb cholesterol far more efficiently than *NPC1b* single mutants. These findings indicate that, while NPC1b normally plays an important role in sterol absorption, there is an *NPC1a*- and *NPC1b*-independent mechanism of sterol absorption in *Drosophila* that functions efficiently in the absence of these factors. Interestingly, the lack of sterol enrichment of midgut tissues that is seen in *NPC1b* mutants is also observed in *NPC1b;NPC1a* double mutants, suggesting that the NPC1a- and NPC1b-independent sterol absorption pathway somehow bypasses sterol enrichment of midgut tissues. One possible explanation of this finding is that the mechanism of the NPC1a- and NPC1b-independent sterol absorption pathway is fundamentally different from that of the NPC1b-dependent pathway and does not require or facilitate sterol enrichment of midgut tissues. Alternatively, the NPC1a- and NPC1b-independent sterol absorption pathway may operate in tissues other than the midgut, such as Malpighian tubules. Identification of the NPC1a- and NPC1b-independent sterol absorption apparatus will be required to resolve these matters.

There are several potential models to explain the effects of mutations in NPC1a on sterol absorption. One possibility is that there is a sterol-sensing mechanism in *Drosophila* that monitors the flux of sterol trafficking intermediates or the abundance of downstream products of sterol trafficking, such as ecdysone. The sensing mechanism then relays this information to regulate sterol absorption through the alternate sterol absorption pathway (Figure 7). Alternatively, loss of NPC1a activity may decrease the efflux of dietary-derived sterols by blocking the trafficking of sterols to the midgut epithelium plasma membrane for delivery to ATP-binding cassette (ABC) family of efflux transporters. These different modes of regulation may act through a cell-autonomous (Figure 7A) or non-cell-autonomous (Figure 7B) mechanism. These models should be readily testable in future experiments.

While our findings indicate that NPC1b promotes an early step in dietary sterol acquisition, several observations suggest that *NPC1b* mutant larvae die for reasons unrelated to sterol deficiency. First, filipin staining of the CNS and Malpighian tubules of *NPC1b* mutants revealed fluorescence intensities comparable to that of wild-type controls (Figure 5 and Figure 6), and the results of a conventional assay to measure total sterol abundance indicated that *NPC1b* mutants are not severely depleted of sterols (Figure S2). Second, unlike the *NPC1a* recessive lethal phenotype, which is strongly influenced by dietary sterols, the *NPC1b* mutant lethal phase is not detectably influenced by increased dietary sterol content. Similarly, increased maternal loading of sterols during oogenesis

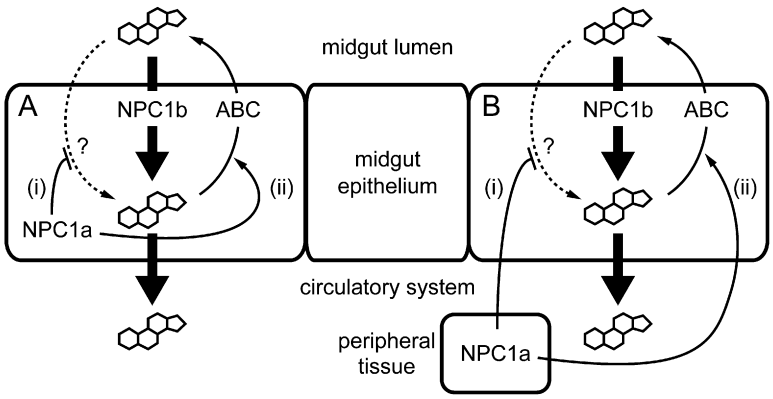

Figure 7. Models to Explain the Roles of NPC1a and NPC1b in Sterol Absorption

All models posit that NPC1b plays an early role in the acquisition of dietary sterols but that an NPC1a- and NPC1b-independent mechanism of sterol absorption (designated by the dotted line) also allows entry of sterols. The regulatory role of NPC1a in sterol absorption may involve inhibition of the NPC1a- and NPC1b-independent mode of sterol absorption under conditions of efficient intracellular sterol trafficking (i). Alternatively, NPC1a may activate the efflux of dietary sterols, perhaps by promoting the trafficking of sterols back to the plasma membrane for delivery to ABC-family transporters (ii). These modes of regulation may involve a cell-autonomous role of NPC1a (A) or a non-cell-autonomous role of NPC1a (B). The NPC1a- and NPC1b-independent mechanism of sterol delivery to the circulatory system may reside within the midgut epithelium, as pictured here, or within another tissue altogether. However, the general models depicted in (A) and (B) are proposed to explain the role of NPC1a in the regulation of dietary sterol absorption, regardless of the tissue in which the NPC1a- and NPC1b-independent sterol absorption apparatus resides.

does not shift the lethal phase of *NPC1b* mutants, in contrast to our results with *NPC1a* mutants. Third, in contrast to our findings in *NPC1a* mutants, exogenous ecdysone is unable to alter the lethal phase of *NPC1b* mutants. This latter finding cannot be readily explained by a defect of *NPC1b* mutants in the absorption of this nutrient from the midgut because previous studies have shown that 20-hydroxyecdysone can be absorbed directly, apparently without need of active transport (Ogiso and Ohnishi, 1984). Based on these findings, we suggest that, while NPC1b promotes the absorption of sterols from the midgut epithelium, the *NPC1b* gene is essential for viability either because defective sterol absorption in the midgut adversely influences an essential process that is unrelated to ecdysone metabolism or because *NPC1b* mutants fail to absorb another unknown essential dietary factor. Further studies will be required to resolve this matter.

In conclusion, our results indicate that the NPC1b protein normally plays an early role in the acquisition of dietary sterols in the midgut epithelium. However, under conditions of defective intracellular sterol trafficking and possibly dietary restriction of sterols, the absorption of sterols increases, apparently through an NPC1a- and NPC1b-independent mechanism. Moreover, our studies raise the possibility that NPC1b also promotes the absorption of one or more essential nonsterol nutrients. While our work advances our current understanding of the molecular mechanisms of sterol absorption, it also raises many new questions—in particular, the possibility of a mechanism by which the efficiency of intracellular trafficking is monitored in insects and conveyed to a novel NPC1-independent sterol absorption apparatus. Our knowledge of the precise mechanism by which NPC1a and NPC1b promote intracellular sterol trafficking and sterol absorption, respectively, and the factors that regulate the expression and function of NPC1a and NPC1b is also far from complete. Further analyses of *Drosophila NPC1a* and *NPC1b* mutants should provide answers to these questions.

EXPERIMENTAL PROCEDURES

Fly Strains and Culture

All marker mutations and balancer chromosomes were as described and referenced by the FlyBase Consortium (2003). Fly stocks bearing transgenes encoding heat-shock-inducible flippase, I-SceI, and I-CreI were obtained from K. Golic (University of Utah). All other fly stocks were obtained from the Bloomington Drosophila Stock Center. Flies were raised on standard cornmeal/molasses food at 25°C unless otherwise noted. Food supplementation experiments were carried out as previously described (Fluegel et al., 2006).

Generation of *NPC1b* Alleles

An *NPC1b* mutant was created using a gene-targeting method by engineering a targeting construct with a stop-codon mutation at codon 392 of the *NPC1b* gene (CG12092), nucleotide alterations designed to eliminate the adjacent 5′ splice site, a diagnostic XhoI restriction site, and other modifications required for gene targeting (Figure 2A; Rong and Golic, 2000). The targeting construct includes a sequence corresponding to nucleotides 273721–280080 of genomic DNA accession number AE003569. Three separate PCR reactions were performed to amplify this genomic region of *NPC1b* while also introducing the inactivating mutations and homing endonuclease recognition sites. All fragments were amplified from w^{1118} genomic DNA using the Taq-Plus Precision system (Stratagene). One fragment was amplified using the primers 5′-AAAAGGTACCGAAGTTGCCGAGCA-3′ and 5′-AAAACTGAGTCATGTCTGATTGACGG-3′, which generate a 2.1 kb product containing the NPC1b-inactivating mutations. Another fragment was amplified using the primers 5′-ACACCTCGAGAGCAAT AAACTAGGCT-3′ and 5′-AAAACTAGTATTACCCTGTTATCCCTACC GGTGGCCAGCAT-3′, which generate a 700 bp product containing an I-SceI homing endonuclease recognition site. The third fragment was amplified using the primers 5′-AAAACTCGAGACCGGACTAGT GCT-3′ and 5′-AAACCGCGGCGGCCGCGATTTTCCTCCAGCTTT-3′, which generate a 3.5 kb product. The fragments were joined in sequential steps using unique restriction sites engineered into the primer sequences, and the proper arrangement was verified by sequencing the junctions. The completed 6.3 kb targeting construct was cloned into a KpnI-NotI-digested pTV2 vector (kindly provided by K. Golic), which supplies a w^{+} marker and FRT sites flanking the targeting construct, and then introduced into the *Drosophila* genome through standard germline transformation techniques (Rubin and Spradling, 1982).

A transgenic line bearing the targeting construct situated on a chromosome with the dominant *Cy* mutation was crossed to flies bearing both flippase and I-SceI transgenes under the control of heat-shock

promoters. Progeny of this cross were heat shocked at 38°C for 1 hr 0–3 days after hatching and grown to adulthood. Expression of flippase in the germline of these flies is expected to excise the targeting construct as a circular fragment, and I-SceI generates a double-stranded break in that fragment to convert the targeting construct to a linear fragment. In rare instances, the linearized targeting construct reinserts into the genome at the location of the homologous genomic locus, typically generating a tandem duplication of the sequence of interest. Putative targeting events were detected by screening for flies bearing the w^+ eye-color marker that also lacked the *Cy* mutation, indicating that the targeting construct mobilized to another chromosome. All putative targeting events displaying an X chromosome segregation pattern of the w^+ marker were subjected to molecular analysis to confirm proper targeting of the X-linked *NPC1b* gene. From among the 35,000 progeny screened, two independent targeting events were identified. Both targeting events consisted of a tandem arrangement of the mutationally altered and wild-type copies of the *NPC1b* gene (Figure 2A).

To convert the tandem pair of *NPC1b* alleles to a single copy of the mutationally altered *NPC1b* allele, the properly targeted flies were crossed to flies bearing an I-CreI transgene under the control of a heat-shock promoter. Progeny of this cross were heat shocked at 38°C for 1 hr 0–3 days after hatching and grown to adulthood. Expression of I-CreI is expected to generate a double-stranded break at the I-CreI recognition site in the targeting transgene, and recombinational repair of this double-stranded break can resolve the tandem duplication to a single copy of the *NPC1b* gene. The desired progeny were detected by screening for the absence of the w^+ eye-color marker (Figure 2A). From among the 400 progeny screened, 33 mutant lines were isolated and verified by the presence of the diagnostic XhoI restriction site (Figure 2B).

Generation of Transgenic Constructs

The *NPC1b* genomic rescue construct was generated by cloning a 6.6 kb XhoI-EagI fragment containing the complete *NPC1b* coding sequence and approximately 0.9 kb of upstream sequence from the BAC clone RPCI-11M14 (BACPAC Resource Center) into a modified form of the *Drosophila* vector pUAST lacking UAS motifs.

The *UAS-NPC1b* transgene was generated from the *NPC1b* genomic rescue transgene. First, to insert a 5′ restriction site at the predicted start of *NPC1b* translation, a 1.1 kb product was amplified from genomic DNA using the primers 5′-ACACCTCGAGCACAACC AAAATGAAAGTGATTTTTGCAAC-3′ and 5′-ACGGCCTTTACGAACA TCTG-3′. This PCR product was cloned into an XhoI-MluI-digested *NPC1b* genomic transgene. The entire *NPC1b* coding sequence was then cloned into a KpnI-EagI-digested pUASp *Drosophila* transformation vector (Rorth, 1998).

The *NPC1b-GAL4* transgene was generated by PCR amplification of a 0.9 kb fragment immediately upstream of the predicted NPC1b start codon with the primers 5′-CAGGTGGTTGGTATTTCTCAG-3′ and 5′-CGCGGATCCTCCGACACTAAAAAAAAAAAAATA-3′. This fragment was then cloned into an XhoI-BamHI-digested pG4PB reporter vector (Dobritsa et al., 2003) (kindly provided by J. Carlson, Yale University). The integrity of all constructs was verified by sequencing before introduction into *Drosophila* by standard germline transformation techniques.

Filipin Staining of Larval Tissues

Larvae were collected from yeasted grape-juice agar plates maintained at 25°C. For filipin staining experiments involving midgut tissues, 12–24 hr old larvae were used. All other filipin staining experiments used 0–4 hr old larvae. Larval tissues were dissected in PBS and fixed in 4% paraformaldehyde in PBS for 20–30 min. After fixation, tissues were washed at least twice with PBS. A fresh 250 μg/ml stock solution of filipin (Sigma) was made daily in DMSO. Tissues were stained with 50 μg/ml filipin in the dark for 30 min at room temperature followed by three washes in PBS. Following staining, tissues were placed in 50% glycerol/PBS overnight at 4°C before mounting onto slides with ProLong Gold Anti-Fade reagent (Invitrogen). Stained midgut tissues were analyzed on a Nikon Microphot-FXA microscope using an excitatory wavelength of 360–370 nm, and the filipin signal was detected at wavelengths above 400 nm. All other filipin-stained tissues were analyzed using a Leica SP1 two-photon confocal microscope. The Tsunami laser produced an excitatory wavelength of 723–741 nm, and the filipin signal was detected at wavelengths of 399–553 nm. Equivalent gain and offset values were used for each tissue type across genotypes.

Cholesterol Absorption Assay

Food for cholesterol absorption studies was prepared by boiling 0.7 g agar, 1 g yeast extract, 2.5 g dextrose, and 1.5 g Nipagin in a total volume of 100 ml of water. Ten microcuries of [1a,2a(n)-^{3}H]cholesterol (Amersham) was evaporated in a fume hood to remove toluene and resuspended in 100% ethanol. Ten microcuries of [1a,2a(n)-^{3}H]cholesterol in 100% ethanol and five microcuries of D-[6-^{14}C]glucose in 3% ethanol (Amersham) were each added to 1 ml of prepared agar along with 100 μl of diluted food coloring. This mixture was then distributed in 200 μl aliquots onto small Petri dishes. Agar plates lacking both radioactivity and food coloring were also prepared. Larvae were collected at room temperature onto wetted 2.5 cm Whatman paper disks from yeasted grape-juice agar plates 3–5 hr after hatching using GFP fluorescence to identify mutants as necessary.

Collected larvae were transferred en masse to food containing radioactive cholesterol and glucose and were incubated for 18 hr at room temperature. Following this incubation period, larvae with colored gut contents were then transferred to nonradioactive agar for 4 hr. Larvae that lacked colored gut contents following this incubation period were then placed into microfuge tubes. One hundred microliters of EcoScint A scintillation fluid (National Diagnostics) was added to larval samples, which were then homogenized using Kontes pestles. This homogenate was then added to a scintillation vial containing 5 ml of scintillation fluid and analyzed in a scintillation counter. Cholesterol absorption experiments were repeated three times in entirety, and the ^{3}H:^{14}C ratios from these experiments were averaged.

Cholesterol turnover was assessed by adding 5 μCi [4-^{14}C]cholesterol (Amersham) in ethanol to agar prepared as described above along with food coloring. Larvae were collected as described above and placed onto radioactive agar for 2 hr. Larvae displaying colored guts were then transferred onto agar plates lacking radioactive cholesterol. After 4 hr, half of the larvae were collected, homogenized, and counted as described above. The remaining larvae were similarly processed after a further 14 hr on nonradioactive agar. The number of counts was compared at the two time points to assess the turnover rate of cholesterol over this time period. Trials were conducted in triplicate for all genotypes analyzed.

Sterol Quantitation Assay

The Amplex Red Cholesterol Assay Kit (Molecular Probes) was used to assess total sterol content. First-instar larvae were genotyped, collected from grape-juice agar plates lacking yeast, and placed on yeast paste for 8 hr to feed. Larvae were then collected, rinsed of all yeast, and transferred to apple-juice agar lacking yeast for 3 hr. Larvae were then weighed and homogenized in 150 mm NaCl, 50 mm Tris (pH 7.5), 2 mm EGTA to make a 100 mg/ml larval homogenate. The homogenate was centrifuged at 5000 rpm for 5 min to pellet debris, and the supernatant was assayed according to manufacturer's instructions. Fluorescence was measured with a Packard FluoroCount fluorometer with a 530/590 nm filter set.

Supplemental Data

Supplemental Data include two figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/5/3/195/DC1/.

ACKNOWLEDGMENTS

The authors express their gratitude to J. Greene and A. Poole for technical help with the creation of the *NPC1b* targeting construct and for

developing our filipin methodology, respectively. The authors also thank K. Golic for helpful discussions on the gene-targeting methodology and for providing reagents, J. Carlson for the pG4PB vector, and all members of the Pallanck lab for insightful input on this project. This work was made possible by grants from the Jim Lambright Niemann-Pick Foundation, the Ara Parseghian Medical Research Foundation, the American Heart Association (AHA 0655717Z to L.J.P. and AHA 0515513Z to M.L.F.), and the National Institutes of Health (F31 GM70186 to S.P.V.).

Received: August 8, 2006
Revised: December 4, 2006
Accepted: January 23, 2007
Published: March 6, 2007

REFERENCES

Altmann, S.W., Davis, H.R., Jr., Zhu, L.J., Yao, X., Hoos, L.M., Tetzloff, G., Iyer, S.P., Maguire, M., Golovko, A., Zeng, M., et al. (2004). Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. Science *303*, 1201–1204.

Brown, M.S., and Goldstein, J.L. (1986). A receptor-mediated pathway for cholesterol homeostasis. Science *232*, 34–47.

Carstea, E.D., Morris, J.A., Coleman, K.G., Loftus, S.K., Zhang, D., Cummings, C., Gu, J., Rosenfeld, M.A., Pavan, W.J., Krizman, D.B., et al. (1997). Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis. Science *277*, 228–231.

Clayton, R.B. (1964). The utilization of sterols by insects. J. Lipid Res. *15*, 3–19.

Davies, J.P., and Ioannou, Y.A. (2006). The role of the Niemann-Pick C1-like 1 protein in the subcellular transport of multiple lipids and their homeostasis. Curr. Opin. Lipidol. *17*, 221–226.

Davies, J.P., Chen, F.W., and Ioannou, Y.A. (2000a). Transmembrane molecular pump activity of Niemann-Pick C1 protein. Science *290*, 2295–2298.

Davies, J.P., Levy, B., and Ioannou, Y.A. (2000b). Evidence for a Niemann-pick C (NPC) gene family: identification and characterization of NPC1L1. Genomics *65*, 137–145.

Davies, J.P., Scott, C., Oishi, K., Liapis, A., and Ioannou, Y.A. (2005). Inactivation of NPC1L1 causes multiple lipid transport defects and protects against diet-induced hypercholesterolemia. J. Biol. Chem. *280*, 12710–12720.

Davis, H.R., Jr., Zhu, L.J., Hoos, L.M., Tetzloff, G., Maguire, M., Liu, J., Yao, X., Iyer, S.P., Lam, M.H., Lund, E.G., et al. (2004). Niemann-Pick C1 Like 1 (NPC1L1) is the intestinal phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. J. Biol. Chem. *279*, 33586–33592.

Dobritsa, A.A., van der Goes van Naters, W., Warr, C.G., Steinbrecht, R.A., and Carlson, J.R. (2003). Integrating the molecular and cellular basis of odor coding in the Drosophila antenna. Neuron *37*, 827–841.

Fluegel, M.L., Parker, T.J., and Pallanck, L.J. (2006). Mutations of a Drosophila NPC1 gene confer sterol and ecdysone metabolic defects. Genetics *172*, 185–196.

FlyBase Consortium (2003). The FlyBase database of the Drosophila genome projects and community literature. Nucleic Acids Res. *31*, 172–175.

Garcia-Calvo, M., Lisnock, J., Bull, H.G., Hawes, B.E., Burnett, D.A., Braun, M.P., Crona, J.H., Davis, H.R., Jr., Dean, D.C., Detmers, P.A., et al. (2005). The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). Proc. Natl. Acad. Sci. USA *102*, 8132–8137.

Garen, A., Kauvar, L., and Lepesant, J.-A. (1977). Roles of ecdysone in *Drosophila* Development. Proc. Natl. Acad. Sci. USA *74*, 5099–5103.

Garver, W.S., and Heidenreich, R.A. (2002). The Niemann-Pick C proteins and trafficking of cholesterol through the late endosomal/lysosomal system. Curr. Mol. Med. *2*, 485–505.

Gilby, A.R. (1965). Lipids and their metabolism in insects. Annu. Rev. Entomol. *10*, 141–160.

Huang, X., Suyama, K., Buchanan, J., Zhu, A.J., and Scott, M.P. (2005). A Drosophila model of the Niemann-Pick type C lysosome storage disease: dnpc1a is required for molting and sterol homeostasis. Development *132*, 5115–5124.

Ioannou, Y.A. (2000). The structure and function of the Niemann-Pick C1 protein. Mol. Genet. Metab. *71*, 175–181.

Iyer, S.P., Yao, X., Crona, J.H., Hoos, L.M., Tetzloff, G., Davis, H.R., Jr., Graziano, M.P., and Altmann, S.W. (2005). Characterization of the putative native and recombinant rat sterol transporter Niemann-Pick C1 Like 1 (NPC1L1) protein. Biochim. Biophys. Acta *1722*, 282–292.

Karten, B., Campenot, R.B., Vance, D.E., and Vance, J.E. (2006). The Niemann-Pick C1 protein in recycling endosomes of presynaptic nerve terminals. J. Lipid Res. *47*, 504–514.

Ko, D.C., Gordon, M.D., Jin, J.Y., and Scott, M.P. (2001). Dynamic movements of organelles containing Niemann-Pick C1 protein: NPC1 involvement in late endocytic events. Mol. Biol. Cell *12*, 601–614.

Li, J., Brown, G., Ailion, M., Lee, S., and Thomas, J.H. (2004). NCR-1 and NCR-2, the C. elegans homologs of the human Niemann-Pick type C1 disease protein, function upstream of DAF-9 in the dauer formation pathways. Development *131*, 5741–5752.

Malathi, K., Higaki, K., Tinkelenberg, A.H., Balderes, D.A., Almanzar-Paramio, D., Wilcox, L.J., Erdeniz, N., Redican, F., Padamsee, M., Liu, Y., et al. (2004). Mutagenesis of the putative sterol-sensing domain of yeast Niemann Pick C-related protein reveals a primordial role in subcellular sphingolipid distribution. J. Cell Biol. *164*, 547–556.

Mellon, S., Gong, W., and Griffin, L.D. (2004). Niemann pick type C disease as a model for defects in neurosteroidogenesis. Endocr. Res. *30*, 727–735.

Murakami, R., Takashima, S., and Hamaguchi, T. (1999). Developmental genetics of the Drosophila gut: specification of primordia, subdivision and overt-differentiation. Cell. Mol. Biol. (Noisy-le-Grand) *45*, 661–676.

Ogiso, M., and Ohnishi, E. (1984). Uptake and metabolism of [3H]ecdysone in cultured ovaries of the silkworm, Bombyx mori. Mol. Cell. Endocrinol. *38*, 13–19.

Perrimon, N., Smouse, D., and Miklos, G.L. (1989). Developmental genetics of loci at the base of the X chromosome of Drosophila melanogaster. Genetics *121*, 313–331.

Rodenburg, K.W., and Van der Horst, D.J. (2005). Lipoprotein-mediated lipid transport in insects: analogy to the mammalian lipid carrier system and novel concepts for the functioning of LDL receptor family members. Biochim. Biophys. Acta *1736*, 10–29.

Rong, Y.S., and Golic, K.G. (2000). Gene targeting by homologous recombination in *Drosophila*. Science *288*, 2013–2018.

Rorth, P. (1998). Gal4 in the Drosophila female germline. Mech. Dev. *78*, 113–118.

Rubin, G.M., and Spradling, A.C. (1982). Genetic transformation of Drosophila with transposable element vectors. Science *218*, 348–353.

Sane, A.T., Sinnett, D., Delvin, E., Bendayan, M., Marcil, V., Menard, D., Beaulieu, J.F., and Levy, E. (2006). Localization and role of NPC1L1 in cholesterol absorption in human intestine. J. Lipid Res. *47*, 2112–2120.

Sym, M., Basson, M., and Johnson, C. (2000). A model for niemann-pick type C disease in the nematode Caenorhabditis elegans. Curr. Biol. *10*, 527–530.

Xie, C., Richardson, J.A., Turley, S.D., and Dietschy, J.M. (2006). Cholesterol substrate pools and steroid hormone levels are normal in the face of mutational inactivation of NPC1 protein. J. Lipid Res. *47*, 953–963.

Yu, L., Bharadwaj, S., Brown, J.M., Ma, Y., Du, W., Davis, M.A., Michaely, P., Liu, P., Willingham, M.C., and Rudel, L.L. (2006). Cholesterol-regulated translocation of NPC1L1 to the cell surface facilitates free cholesterol uptake. J. Biol. Chem. *281*, 6616–6624.